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Complete List of Authors:	Yao, Kang; Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Fudan University, Department of Cardiology Wang, Yanzhong; Kings College London, School of Population Health and Environmental Sciences Liu, Xuebo; Tongji Hospital, Tongji University, Department of Cardiology shen, chengxing; Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Department of Cardiology Hu, Wei; Minhang Hospital, Fudan University, Department of Cardiology Wang, Zhe; Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Fudan University, Department of Cardiology Wu, Runda; Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Fudan University, Department of Cardiology Tang, Xianglin; Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Fudan University, Department of Cardiology Sun, Aijun; Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Fudan University, Department of Cardiology; Shanghai Institute of Biomedical Science, Fudan University Zou, Yunzeng; Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Fudan University, Department of Cardiology Qian, Juying; Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Fudan University, Department of Cardiology Wu, Guangyu; Qlife Lab Co., Ltd. Guo, Xin; Qlife Lab Co., Ltd. Cheng, Xiaoliang; Qlife Lab Co., Ltd. Ge, Junbo; Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Fudan University, Department of Cardiology
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The Effect of Combined Testing of Ceramides with High-sensitive Troponin T on the Detection of Acute Coronary Syndrome in Patients with Chest Pain in China： a prospective observational study

Authors: Kang Yao[1*], MD; Yanzhong Wang[2*], PhD; Xuebo Liu[3], MD; Cheng-Xing Shen[4], MD; Wei Hu[5], MD; Zhe Wang[1]; Runda Wu[1], MD; Xianglin Tang[1], MD; Aijun Sun[1,6], MD; Yunzeng Zou[1], MD; Juying Qian[1], MD; Guangyu Wu[7], MD, PhD; Xin Guo[7], MD; Xiaoliang Cheng[7], PhD; Junbo Ge[1], MD

[*] Drs Yao and Wang contributed equally.

1. Department of Cardiology, Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Fudan University, Shanghai, China
2. School of Population Health and Environmental Sciences, King's College London, London, UK
3. Department of Cardiology, Tongji Hospital, Tongji University, Shanghai, China
4. Department of Cardiology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China
5. Department of Cardiology, Minhang Hospital, Fudan University, Shanghai, China
6. Institute of Biomedical Science, Fudan University, Shanghai, China
7. Qlife Lab Co., Ltd., 700 Yishan Road, Shanghai Putian Information Industrial Park, Shanghai, China

Corresponding author:

Prof. Junbo Ge, MD, FACC, FESC

Department of Cardiology, Shanghai Institute of Cardiovascular Diseases,

Zhongshan Hospital, Fudan University

180 Fenglin Road, Shanghai, China. Postcode: 200032

Tel: 0086 (0)21 64041990-2152; Fax: 0086 (0)21 64437078

Email: ge.junbo2@zs-hospital.sh.cn

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Abstract

Objective Ceramides are associated with coronary plaque vulnerability. We aim to investigate the potential diagnostic value of ceramides for Acute Coronary Syndrome (ACS) in Chinese patients with chest pain.

Design Prospective observational survey.

Setting Shanghai, China, 2016-7.

Participants 2773 patients with chest pain from four hospitals in Shanghai, China between August 2016 and October 2017.

Main outcome measures Performance of metabolites model in detection of ACS cases including ST-elevation myocardial infarction (STEMI), non-ST-elevation myocardial infarction (NSTEMI), and unstable angina (UA).

Results – Plasma levels of 12 ceramides molecules and corresponding ratios were compared between patients diagnosed with ACS and those without. Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0) ratio, Cer(d18:1/14:0) and Cer(d18:1/22:0) were independent predictors of ACS after adjustment of traditional risk factors and high sensitive-cTnT. Receiver operating characteristic curve (ROC) analysis showed a significant improvement in detecting ACS in the multi-variable model with ceramides as compared to that without [0.865 (0.840-0.889) *vs.* 0.808 (0.776-0.841), $P < 0.001$].

Conclusion – Distinct plasma ceramides are independent diagnostic predictors of ACS among patients with chest pain. Ceramides together with high-sensitive troponin and traditional factors showed great potential in identifying ACS among patients with chest pain.

Article Summary

Strengths and limitations of this study

- This is the first study with systematic demonstration of plasma levels of 12 ceramide molecules in 2773 Chinese patients with chest pain.
- Performance of ceramides levels in diagnosing acute coronary syndrome was assessed and validated in multi-center clinical studies.
- The time of ceramides level elevation after ACS occurrence cannot be assessed in the study design.

1. Introduction

Acute Coronary Syndrome (ACS), which includes both myocardial infarction (MI) and unstable angina (UA), is estimated to affect 1.4 million people in the United States, and 2.5 million people in China per year.[1 2] However, patients with acute chest pain suggestive of ACS present with a heterogeneous array of conditions, including both non-ischaemic and ischemic chest pain.[3] Only 17% of these are finally diagnosed as ACS caused by atherosclerotic and ischaemic heart disease.[3]

The likelihood of ACS in patients with chest pain is estimated via the entire clinical picture, including symptoms and physical examination findings, disease history, electrocardiogram (ECG) changes, and biomarkers results. Among biomarkers, cardiac troponins play a central role in establishing a diagnosis and stratifying risk. Troponins are more specific and sensitive than the traditional cardiac enzymes such as creatine kinase (CK), its isoenzyme MB (CK-MB), and myoglobin.[4] However, there has been an increasing recognition of other biomarkers recently.[5] Considerable effort has been made to improve the multi-biomarker based evaluation and management of ACS. Altered lipid metabolism associated with inflammation and oxidative stress initiate the pathological changes, including the formation of lipid-laden foam cells and the necrotic lipid core of unstable plaque.[6 7] As many lipid types are essential in atherogenesis, they should be used for ACS prediction.[6]

Ceramides are a family of lipid molecules that are found in high concentrations within cell membranes and play a key role in a variety of physiology functions including apoptosis, cell growth, cell adhesion and plasma membrane integrity maintenance.[8] All tissues can synthesize ceramides de novo from saturated fats and sphingosine.[9] However, lesional low density lipoprotein (LDL) is known to be rich in ceramide in the atherosclerotic plaque, and it contains 10- to 50-fold-higher content of ceramide when compared with plasma LDL.[10] An arterial-wall sphingomyelinase (SMase) hydrolyzes the sphingomyelin (SM) of retained LDL.[10] Plasma ceramide (d18:1/16:0), is associated with the fraction of necrotic core tissue and lipid core burden in coronary atherosclerosis.[11] Inflammatory cytokine tumor necrosis factor alpha was shown to induce the production of ceramide in vascular endothelial cells,[12 13]

via the activation of SMase, accompanied by reactive oxygen species production.[14] High content of oxidized LDL (ox-LDL) in the plaque is capable of activating precursors to matrix metallopeptidase 2, propagating the signalling of the ceramide pathway by activation of SMase.[15] These findings suggest abnormal regulation of ceramides might be related to plaque instability and triggering of ACS.

Previous clinical research found that circulating ceramides are elevated in patients with hypertension,[16] obesity,[17] [18] and type 2 diabetes mellitus.[18] Ceramides ratio levels such as Cer(d18:1/16:0) / Cer(d18:1/24:0) ratio, Cer(d18:1/18:0) / Cer(d18:1/24:0) ratio, and Cer(d18:1/24:1) / Cer(d18:1/24:0) ratio were proposed as useful biomarkers for cardiovascular death (CV death) prediction, as assessed by three independent coronary artery disease (CAD) cohorts.[19]

Although several studies have shown plasma levels of specific ceramide molecules are correlated with diagnosis of patients with cardiac events, no studies to date has evaluated the role of these ceramide molecules in detecting ACS from patients with chest pain. In this study, we aim to assess the value of ceramides in detection of ACS in patients with chest pain and whether a combination of ceramides and troponin could improve the diagnostic power.

2. Methods

2.1. Study design and participants

This is a prospective observational study involving four University affiliated hospitals in Shanghai, China. A total of 2990 patients with chest pain were consecutively recruited in chest pain outpatient during Monday to Friday between August 2016 and October 2017, and 2773 patients were finally admitted to wards of cardiology after pre-screening by the exclusion criteria. Sample size calculation is shown in Supplementary File. Patients were eligible if they were aged ≥18 and ≤ 80 years, presented with signs and symptoms of chest pain and agreed to participate in this study. The exclusion criteria were pregnant women; organ transplant patients; patients suffering from bleeding disorders; patients

with neoplasms with a life expectancy <1 year; chronic kidney disease (eGFR < 60 mL/min/1.73m^2) and patients with a clear non-cardiac chest pain. A flow chart describing the inclusion and exclusion process is shown as Figure 1. Full study protocol is provided upon request to the corresponding author. The study complies with the Declaration of Helsinki and was approved by the Ethics Committee of Zhongshan Hospital, the Ethics Committee of Tongji Hospital, the Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital, and the Ethics Committee of Minhang Hospital. Written informed consent was obtained from all participants.

2.2. Clinical and laboratory data

Clinical history, physical examination findings and blood tests including renal and liver functions, C-reactive protein (CRP), D-dimer and high-sensitive cTnT (Roche) levels, ECG, coronary angiogram and echocardiogram findings were recorded in all patients. Troponin levels were measured by electrochemiluminescence method using high sensitive-cTnT assay (Roche Diagnostics) on Roche Cobas e601. The coefficient of variation in the hs-cTnT assay is ⩽10% at the cut-off value of 13 ng/L. The 99 percentile upper reference limit of hs-cTnT assay is 14 ng/L. Besides, the assay also has a limit of blank of 3 ng/L and a limit of detection of 5 ng/L, and the analytical range is 3-10000 ng/L. Regular blood lipid tests were conducted using standard methods. Specifically, total cholesterol (TC) was measured by enzymatic cholesterol method using cholesterol oxidase/peroxidase aminophenazone (COD-PAP) reagent while total triglyceride (TG) was measured by Glycerol-3-Phosphate oxidase/peroxidase anti-peroxidase method (GPO-PAP) method. Low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were measured by homogenous PEG modification enzyme method. Other blood biomedical assay of apolipoprotein (AI, AII, B, and E), lipoprotein (a), and lipoprotein-associated phospholipase were performed by immune transmission turbidity method. Performers of the above tests were blinded to the diagnosis of diseases. The detection limit, analytical range, and reference interval or decision limit are summarized in Supplementary Table 1.

2.3. Diagnosis of ACS

The diagnosis of ACS including UA, Non-ST segment elevation myocardial infarction (NSTEMI), and ST-segment elevation myocardial infarction (STEMI) was made by two independent cardiologists by reviewing all patients' notes, including symptoms, 12-lead ECG, and blood tests results according to the 2014 American Heart Association /American College of Cardiology guidelines and international definition of myocardial infarction version 3. Type 1 and type 2 MI were both included.[20 21] The agreement level and kappa value between the 2 cardiologists were 0.99 and 0.98. The cases where the 2 cardiologists disagreed were reviewed by a senior cardiologist. All the cardiologists were blinded to ceramide levels. Cardiac chest pain with persistent ECG findings of elevation in the ST segment indicate STEMI. Cardiac chest pain and elevations in troponins levels without ST elevation indicate NSTEMI. Those with presence of 1 or more of 3 principal ischemic symptoms ((1) rest angina (lasting >20 minutes), (2) new-onset (<2 months previously) severe angina, and (3) a crescendo pattern of occurrence (increasing in intensity, duration, frequency, or any combination of these factors)) without elevations in cardiac troponins are defined as UA.[5] Participants were diagnosed with ACS when there is evidence of UA, NSTEMI or STEMI.

2.4. Quantification of plasma ceramides

Potential ceramides were generated from previous untargeted and targeted ceramide profile studies.[22 23] Analyses of 12 plasma ceramides were performed using a Waters Xevo TQ-S mass spectrometer (Manchester, UK) equipped with Waters Acuity UPLC I-Class (Milford, MA, USA) in a hospital and Qlife lab collaborative laboratory based in Department of Cardiology. The mass spectrometry was operated in multiple-reaction monitoring (MRM) mode with ESI-positive ionization. The capillary voltage was set at 3.0 kV. and the source temperature was 120℃. The desolvation temperature and gas flow were 400℃ and 800L/h, respectively. The source offset was maintained at 60V.

Blood samples for ceramides test were collected and centrifuged using ethylene diamine tetraacetic acid (EDTA) anticoagulation tube at admission. Plasma were immediately stored in -80℃ for future analysis. Ceramides test were taken immediately after samples of all patients were collected. Before analysis, the samples were thawed at room temperature, then a volume of 800 µl of protein precipitation solution (isopropanol) that containing D7-Cer d18:1/16:0 (0.01 pmol/µl), D7-Cer d18:1/18:0 (0.005 pmol/µl), D7-Cer d18:1/24:0 (0.015 pmol/µl) and D7-Cer d18:1/24:1 (0.015 pmol/µl) was pipetted into 1.5 mL Eppendorff tube after addition of 50 µL of plasma sample. The mixture was thoroughly vortexed for 10 min followed by 5-min centrifugation at 14,000 rpm. Supernatant (70 µL) was collected for LC/MS/MS analysis. Ceramides ratios were calculated using Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/24:1(15Z)) and Cer(d18:1/24:0).[23] Lab technicians who were in charge for ceramides test were blinded to disease diagnosis.

2.5. Statistical methods

Continuous variables are summarized as mean (standard deviation) or median (interquartile range) and categorical data as count (percentage). Student's t-test, Mann-Whitney U, and Wilcoxon signed rank tests were used to test differences in continuous variables where appropriate, and Chi-squared test was used for proportions. Univariate logistic regression was used to analyse the association of each variable and binary outcome. Multivariable logistic regression analyses were undertaken to identify the demographic, clinical and laboratory factors associated with ACS in patients with chest pain after adjusting for potential confounders and the final models included variables significantly associated with ACS.

Training and validation cohorts were developed via a 10-fold cross-validation approach repeated 10 times.[24] The estimate of prediction error is almost unbiased under 10-fold cross-validation. Repeated 10 times that minimizes the cross-validated error is then used to build the final model.[25] [26] The performance and discrimination ability of the five models were assessed using the area under the

receiver operating curve (AUROC) and net reclassification index (NRI). ROC curves and NRI values were compared among models in training and validation datasets.

All tests were two-tailed, and *P* values less than 0.05 were considered statistically significant. Odds ratios (OR) with 95% confidence intervals (CIs) for the chosen clinical and laboratory variables in the logistic regression models and AUROC and NRI values with 95% CIs for different test datasets were calculated. Statistical analyses were done with statistical software R, version 3.4.3.

2.6.Patient and Public Involvement

This study was conducted without patient and public involvement.

3. Results

3.1. Demographics, lipid profiles and laboratory findings of patients

The demographics, clinical and lab findings of all participants are reported in Table 1. Serial troponin levels were measured at 0h-3h after admission in all recruited patients and all patients underwent echocardiography and coronary angiography. In 2773 patients with chest pain, 354 (12.8%) were diagnosed with ACS, among whom 116 (4.2%) were UA, 114 (4.1%) were NSTEMI, and 124 (4.5%) were STEMI. All 116 patients with UA had a significant stenosis on coronary angiography. Compared to those without ACS, patients with ACS are more likely to be men (73.44% vs 67.57%), to be current smokers (31.35% vs 21.62%), and to have hypertension (68.92% vs 62.00%). Lipid profiles and lipoproteins including TG, TC, LDL-C, ApoB, ApoE, Lipoprotein(a) (Lp(a)) were significantly elevated in patients diagnosed with ACS while HDL-C and apolipoprotein A1 (ApoA1) were significantly lower in ACS patients (all $p<0.001$, except for Lp(a), $p=0.023$). CRP and cardiac damage markers including hs-cTnT and N-terminal pro-brain natriuretic peptide (NTproBNP) levels were significantly higher in ACS patients (all $p<0.001$). Demographics, lipid profiles and laboratory findings of patients diagnosed with STEMI, NSTEMI and UA were also reported in Supplementary Table 2.

3.2. Plasma ceramides levels in ACS patients

Table 2 presents the levels of twelve ceramides molecules in patients with and without ACS. The levels of 12 ceramide molecules (Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/24:0), Cer(d18:1/24:1(15Z)), Cer(d18:1/14:0), Cer(d18:1/20:0), Cer(d18:1/22:0), Cer(d18:0/16:0), Cer(d18:0/18:0), Cer(d18:0/24:0), Cer(d18:0/24:1(15Z)), Cer(d18:1/24:1)) were all significantly elevated in patients with ACS compared to those without (all $p<0.001$, except for Cer(d18:1/24:0), $p=0.028$) (Table 2). In addition, 3 ceramide ratios (Cer(d18:1/16:0)/Cer(d18:1/24:0), Cer(d18:1/18:0)/Cer(d18:1/24:0) and Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0)) were also significantly increased in ACS patients with all p values less than 0.001. In patients diagnosed with UA and NSTEMI, levels of ceramides and ceramide ratios were also significantly higher compared to patients diagnosed with non-ACS (Supplementary Table 3).

3.3. Association between plasma ceramides and traditional and cardiac damage markers

The associations between plasma ceramides with traditional ACS risk factors and cardiac markers for myocardial injury are shown in Supplementary Table 4. Most of the 12 ceramide molecules and ceramide ratios were moderately correlated with lipid profiles including TG, TC, LDL-C, ApoB and ApoE, while CRP was shown to be weakly correlated with ceramides. There was no significant correlation between ceramides and myocardial damage markers including hs-cTnT and NTproBNP.

3.4. Clinical predictors and multi-variable models for ACS

Clinical predictors for ACS were identified in univariate logistic regression analysis and are shown in Table 3. Being male, having hypertension, and being current smoker were significantly associated with ACS ($p<0.05$). Besides, most of the lipid profile (TG, TC, HDL-C, LDL-C, ApoA1 and ApoB) were significantly associated with ACS (all $p<0.001$). Also, increased levels of hs-cTnT (OR and 95%CI: 2.45 [2.23-2.70]), NTproBNP (1.73 [1.60-1.87]) and CRP (1.73 [1.58, 1.90]) were significantly

associated with ACS. Furthermore, all 12 ceramides levels and 3 ceramides ratios were significantly associated with ACS with all p<0.001 except Cer(d18:1/24:0) with p=0.025 (Table 3).

Multivariable logistic regression analyzing independently significant predictors of ACS are shown in Table 4. Model 1 included traditional risk factors such as hypertension, current smoker, CRP, LDL-C and NTproBNP (all p<0.001 except for hypertension p=0.033). Model 2 only included hs-cTnT (2.454 [2.239-2.701], p<0.001). Model 3 included traditional risk factors and hs-cTnT. Current smoker, CRP, LDL-C, NTproBNP as well as hs-cTnT were independently significant in detecting patients with ACS. Model 4 included traditional risk factors and ceramides levels, with OR (95%CI) of Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0): 2.960 (2.156-4.072), Cer(d18:1/14:0): 1.439 (1.007-2.062) and Cer(d18:1/22:0): 2.079 (1.315-3.288). Model 5 included traditional risk factors, hs-cTnT (2.190, [1.939-2.485]) and ceramides levels (Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0): 4.455 [3.112-6.396], Cer(d18:1/14:0): 1.73 [1.166-2.605], and Cer(d18:1/22:0): 2.301 [1.371, 3.866], all p<0.01). Ceramides were shown to be independently associated with ACS after adjusting for traditional factors and hs-cTnT.

3.5. Discrimination and reclassification of ACS with various multi-variable logistic regression models

As shown in Table 5, the model including traditional factors (hypertension, current smoker, LDL-C, CRP and NT-proBNP level) had an area under the curve (AUC) of 0.780 (0.750-0.811) in diagnosing ACS. AUC of hs-cTnT in predicting ACS was 0.800 (0.767-0.833). After combining traditional factors and hs-cTnT, the AUC became 0.808 (0.776-0.841) (Figure 2A). The AUC of model including traditional factors and ceramides was 0.811 (0.784-0.837). Notably, when combining traditional factors, hs-cTnT, and ceramides, the AUC of the model rose to 0.865 (0.840-0.889) (Figure 2B). We also compared discrimination and reclassification power of these models. Results showed that ceramides significantly improved the AUC of the model with traditional factors and hs-cTnT (0.865 [0.840-0.889] *vs.* 0.808[0.776-0.841], p<0.001). Net reclassification index (NRI) analyses also showed that the model

including traditional factors and hs-cTnT was significantly improved by the inclusion of ceramides (NRI=0.511 (0.388-0.635), p<0.001).

4. Discussion

In this study of 2773 Chinese patients with chest pain from four hospitals in Shanghai, China, levels of 12 ceramides were systematically evaluated together with that of traditional risk factors and high-sensitive troponin. Ceramides and their ratios were shown to be independent predictors of ACS in patients with chest pain after adjustment for traditional risk factors (e.g., age, sex, body mass index (BMI), smoking status, and blood cholesterol) and cardiac damage biomarkers (i.e., high-sensitive troponin). The proposed model with ceramides showed substantial promise and improved value as a risk evaluation tool for ACS with improved performance on model discrimination and reclassification. With targeted liquid chromatography–mass spectrometry based lipidomic approach, we have successfully established the characteristics of cardiac risk-related ceramides from 2773 Chinese patients with chest pain and confirmed our hypothesis that plasma levels of ceramide subspecies correlate with atherosclerotic plaque instability and hence might be used as lipidomic markers for ACS.

This is to our knowledge the first study with systematic demonstration of plasma levels of 12 ceramide molecules in a Chinese population. Also, the selection of two ceramides molecules and one ceramide ratio as potential biomarkers for ACS has not been reported before. Although our ceramides-based diagnostic model showed great potential in identifying ACS among patients with chest pain, its clinical utility especially regarding rule-in and rule-out strategies and performances still need to be further investigated and validated to make it fully applicable in clinical settings. We were also restricted for not measuring ceramides levels in a serial pattern and lacking information about the relationships between ceramides levels and time from the onset of ACS. The study was possibly also limited by a potential selection bias for patients with higher risk of ACS during recruitment process from admitted patients rather than patients in emergency departments. In addition, the results found in our study need

to be validated in independent external cohorts. An improvement in the test technique of ceramides, including standardization of test protocol and automated pretreatment of samples is also needed to fulfil the requirement of clinical practice.

Our findings of two ceramides molecules (Cer(d18:1/14:0), Cer(d18:1/22:0)) and one ceramide ratios (Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0)) as diagnostic biomarkers for ACS among patients with suspected symptoms have never been reported before. In addition, only relatively modest correlations between ceramides and lipid profiles including TG, TC, LDL-C, ApoB and ApoE were detected and there was no significant correlation between ceramides with hs-cTnT, NT-proBNP and CRP levels, suggesting that the diagnostic value of ceramides for ACS might be independent of the above laboratory variables. Moreover, AUROC of multi-variable models showed significant improvement of ceramides on traditional risk factors and high-sensitive troponin T. This result provides evidence that distinct ceramide species serve as independent predictors for the risk of ACS, in addition to conventional blood biomarkers such as HDL-C and CRP levels. Ceramides measurement in high-throughput quality-controlled environments is straightforward.[19] By setting up clinical laboratories equipped with robotized sample handling systems and mass spectrometry equipment, it would be feasible to identify patients with chest pain at high cardiovascular risk using our ceramides-based diagnostic model. [19]

Previous clinical research found that elevated ceramide plasma concentrations are associated with coronary plaque vulnerability evaluated by intravascular ultrasound virtual histology and near-infrared spectroscopy.[11] Untargeted lipidomic analyses have identified CAD risk-related ceramide molecules (Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/24:1)) and their ratios with Cer(d18:1/24:0) as potential risk stratifiers for CAD patients.[22] A previous study applying ceramides to the prediction of cardiovascular death from patients with CAD showed that ceramides, independent of other lipid markers and CRP, were significantly associated with CV death.[19] In a recent study including 495 participants with coronary angiography and followed up for 18 years, ceramides were not associated with CAD but were independently associated with major adverse cardiovascular events.[23] A weighted

sum of ceramides including Cer22:0 and Cer24:1 was also reported to be associated with a 2.18-fold higher risk of cardiovascular disease, which was similar with our results.[27] Recent studies showed targeted profiling of ceramides predicted short-term and long-term major adverse cardiac events (MACE).[28 29] Moreover, a consistent increase in ceramide levels and overexpression of 3 enzymes in ceramide biosynthesis were found on rat ischemic myocardium, which is consistent with the elevated plasma levels of ceramides found in our cohort.[29] Nevertheless, no study has identified the diagnostic value of ceramides in patients with chest pain, while we develop models with lipidomic markers for ACS with additional value upon conventional factors and troponin.

Currently, the standard process for the diagnostic assessment of ACS in patients with suspected symptoms relies on clinical evaluation and high-sensitive troponin tests.[4 20] However, high-sensitive troponin assays identify a larger number of patients with elevated troponin results but without a final diagnosis of ACS, making interpretation of test results challenging.[30] The adoption of highly sensitive assays for ACS detection should be accompanied by serial tests and implementation of algorithms, leading to a longer period of observation[31]. In the current settings of Chinese hospitals, self-paying patients with chest pain tend to be over-prescribed by cardiologists with invasive and expensive tests such as coronary angiography. Noteworthily, ceramides levels of patients with UA are higher than those without ACS, which indicates that ceramides may help in identifying patients with UA and higher risk of ACS from populations without significant elevation of cardiac troponins. Moreover, ceramides levels were higher in NSTEMI patients compared to non-ACS ones, indicating a possible role of ceramides in distinguishing between patients with elevated troponins caused by ACS or non-ACS conditions. Nevertheless, the potential roles of ceramides in clinical diagnosis of UA and NSTEMI need further independent investigation and validation.

In conclusion, we have identified ceramides as independent biomarkers associated with ACS and developed a ceramides-based model to evaluate ACS risk among patients with chest pain. The model showed good model discrimination and net reclassification improvement as compared to other models.

Our study characterises ceramides species in Chinese patients with chest pain and demonstrates that ceramides are of diagnostic value and have the potential for detecting ACS, in addition to currently used lipid markers and high-sensitive troponin.

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Author Contributions: Study concept and design (GJ, YK, WY); recruitment of patients and acquisition of samples (YK, LX, SC, HW, WZ, WR, TX, SA, ZY, QJ); analysis and interpretation of data (WY, WG, GX, CX); drafting first version of the manuscript (WG, WY); critical revision of the manuscript and approval of final version (all authors).

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Conflict of interest: Q-life lab holds patents for the diagnostic use of ceramides for cardiovascular risk determination in China. WG, GX and CX work for Q-life and GX and CX are also shareholders. Other authors declare no conflict of interest.

Patient consent: Obtained.

Ethics approval: This study was approved by the Ethics Committee of Zhongshan Hospital, the Ethics Committee of Tongji Hospital, the Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital, and the Ethics Committee of Minhang Hospital.

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Table 1. Demographics, clinical and lab findings of included patients in total cohort.

Variables	Total cohort (n=2773)	non-ACS (n=2419)	ACS (n=354)	*P*
Age (years)	63.5 (9.6)	63.4 (9.4)	63.8 (10.6)	0.515
Male, n (%)	1894 (68.3)	1634 (67.6)	260 (73.4)	0.031
Diabetes, n (%)	689 (24.8)	600 (24.8)	89 (25.1)	0.943
Hypertension, n (%)	1744 (62.9)	1500 (62.0)	244 (68.9)	0.014
Current Smoker, n (%)	634 (22.9)	523 (21.6)	111 (31.4)	<0.001
TG (mmol/L)	1.46 (1.03-2.10)	1.43 (1.01-2.07)	1.61 (1.16-2.23)	<0.001
TC (mmol/L)	3.78 (3.17-4.59)	3.74 (3.13-4.49)	4.28 (3.51-5.01)	<0.001
HDL-C (mmol/L)	1.09 (0.90-1.30)	1.09 (0.91-1.31)	1.01 (0.88-1.23)	<0.001
LDL-C (mmol/L)	1.93 (1.40-2.61)	1.88 (1.36-2.53)	2.42 (1.77-3.08)	<0.001
ApoA1 (g/L)	1.32 (1.16-1.51)	1.33 (1.17-1.52)	1.24 (1.08-1.43)	<0.001
ApoB (g/L)	0.73 (0.58-0.91)	0.72 (0.57-0.89)	0.84 (0.67-1.03)	<0.001
ApoE (mg/L)	38.0 (30.0-47.0)	37.0 (30.0-46.4)	41.0 (32.0-51.0)	<0.001
Lp(a) (mg/L)	125.0 (53.0-290.3)	123.0 (51.0-282.0)	152.0 (65.0-329.0)	0.023
NTproBNP (ng/L)	100.0 (44.7-268.1)	87.4 (42.3-220.3)	364.1 (113.4-1264.0)	<0.001
CRP (mg/L)	1.7 (0.7-5.5)	1.5 (0.6-4.8)	5.4 (1.8-13.9)	<0.001
hs-cTnT (ng/L)	9 (5-16)	8 (5-13)	71 (11-474)	<0.001

Data are median (interquartile range) or count (percentage).

TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; ApoE, apolipoprotein E; LP(a), lipoprotein(a); NTproBNP, N-terminal pro-brain natriuretic peptide; CRP, C-reactive protein; hs-cTnT, high-sensitivity cardiac troponin T.

P value for the difference between patients diagnosed as ACS and non-ACS.

Table 2. Plasma levels of ceramides molecules and ratios in total cohort and by ACS status.

Ceramides (μmol/L)	Total cohort (n=2773)	non-ACS (n=2419)	ACS (n=354)	*P*
Cer(d18:1/16:0)	0.203 (0.165-0.248)	0.198 (0.162-0.241)	0.237 (0.203-0.286)	<0.001
Cer(d18:1/18:0)	0.051 (0.037-0.069)	0.049 (0.036-0.065)	0.068 (0.051-0.084)	<0.001
Cer(d18:1/24:0)	2.065 (1.607-2.680)	2.053 (1.592-2.672)	2.164 (1.675-2.757)	0.028
Cer(d18:1/24:1(15Z))	0.583 (0.448-0.767)	0.569 (0.436-0.739)	0.730 (0.557-0.950)	<0.001
Cer(d18:1/14:0)	0.0027 (0.0020-0.0036)	0.0027(0.0020-0.0035)	0.0030 (0.0024-0.0040)	<0.001
Cer(d18:1/20:0)	0.053 (0.040-0.067)	0.051 (0.039-0.065)	0.063 (0.051-0.078)	<0.001
Cer(d18:1/22:0)	0.381 (0.300-0.490)	0.375 (0.294-0.480)	0.416 (0.336-0.540)	<0.001
Cer(d18:0/16:0)	0.010 (0.007-0.014)	0.010 (0.007-0.013)	0.011 (0.008-0.015)	<0.001
Cer(d18:0/18:0)	0.005 (0.003-0.008)	0.005 (0.003-0.007)	0.007 (0.004-0.011)	<0.001
Cer(d18:0/24:0)	0.061 (0.042-0.086)	0.060 (0.042-0.086)	0.068 (0.048-0.094)	<0.001
Cer(d18:0/24:1(15Z))	0.029 (0.020-0.044)	0.028 (0.019-0.041)	0.040 (0.026-0.058)	<0.001
Cer(d18:1/24:1)	0.262 (0.195-0.375)	0.258 (0.192-0.372)	0.298 (0.221-0.386)	<0.001
Ceramides Ratio				
Cer(d18:1/16:0)/Cer(d18:1/24:0)	0.096 (0.080-0.116)	0.095 (0.079-0.114)	0.107 (0.090-0.132)	<0.001
Cer(d18:1/18:0)/Cer(d18:1/24:0)	0.024 (0.019-0.032)	0.023 (0.018-0.031)	0.031 (0.023-0.039)	<0.001
Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0)	0.269 (0.214-0.360)	0.261 (0.211-0.345)	0.321 (0.253-0.426)	<0.001

Data are median (interquartile range).

P value for the difference between patients diagnosed as ACS and non-ACS.

Table 3. Univariate logistic regression showing significant predictors of ACS in patients with chest pain.

Variables	Total Cohort (n=2773) OR (95%CI)	*P*
Age	1.00 (0.99-1.02)	0.476
Gender	1.33 (1.04-1.71)	0.027
Diabetes	1.02 (0.78-1.31)	0.890
Hypertension	1.36 (1.07-1.73)	0.012
Current Smoker	1.66 (1.29-2.11)	<0.001
TG*	1.40 (1.15-1.70)	<0.001
TC*	5.17 (3.30-8.15)	<0.001
HDL-C*	0.44 (0.30-0.66)	<0.001
LDL-C*	3.14 (2.37-4.17)	<0.001
ApoA1*	0.17 (0.09-0.29)	<0.001
ApoB*	2.83 (2.05-4.01)	<0.001
ApoE*	0.93 (0.76-1.17)	0.534
Lp(a)*	1.10 (1.00-1.21)	0.055
NTproBNP*	1.73 (1.61-1.88)	<0.001
CRP*	1.74 (1.59-1.90)	<0.001
hs-cTnT*	2.45 (2.24-2.70)	<0.001
Cer(d18:1/16:0)*	7.53 (5.11-11.17)	<0.001
Cer(d18:1/18:0)*	4.67 (3.55-6.17)	<0.001
Cer(d18:1/24:0)*	1.41 (1.05-1.90)	0.025
Cer(d18:1/24:1(15Z))*	5.09 (3.79-6.87)	<0.001
Cer(d18:1/14:0)*	2.04 (1.59-2.62)	<0.001
Cer(d18:1/20:0)*	4.55 (3.35-6.22)	<0.001
Cer(d18:1/22:0)*	2.36 (1.75-3.18)	<0.001
Cer(d18:0/16:0)*	2.02 (1.61-2.55)	<0.001
Cer(d18:0/18:0)*	2.26 (1.92-2.66)	<0.001
Cer(d18:0/24:0)*	1.49 (1.21-1.84)	<0.001
Cer(d18:0/24:1(15Z))*	2.73 (2.24-3.33)	<0.001
Cer(d18:1/24:1)*	1.57 (1.26-1.96)	<0.001
Cer(d18:1/16:0)/Cer(d18:1/24:0)*	4.11 (2.87-5.89)	<0.001
Cer(d18:1/18:0)/Cer(d18:1/24:0)*	3.84 (2.93-5.05)	<0.001
Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0)*	2.63 (2.08-3.34)	<0.001

Data are odds ratio (OR) (95%CI). *Log transformed before analysis.

TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; ApoE, apolipoprotein E; LP(a), lipoprotein(a); NTproBNP, N-terminal pro-brain natriuretic peptide; CRP, C-reactive protein; hs-cTnT, high-sensitivity cardiac troponin T; Cer, ceramides.

Table 4. Multivariable logistic regression showing significant predictors of ACS in patients with chest pain after adjustment for conventional risk factors.

Baseline variables	Model 1	Model 2	Model 3	Model 4	Model 5
Hypertension	1.37 (1.03-1.83)*	—	1.35 (0.98-1.87)	1.41 (1.06-1.90)*	1.40 (1.01-1.96)*
Current Smoker	1.93 (1.43-2.58)‡	—	1.52 (1.09-2.10)*	1.99 (1.47-2.69)‡	1.58 (1.12-2.21)†
LDL-C§	2.63 (1.91-3.64)‡	—	2.01 (1.42-2.87)‡	2.13 (1.51-3.04)‡	1.64 (1.12-2.41)*
CRP§	1.40 (1.27-1.55)‡	—	1.20 (1.07-1.34)†	1.38 (1.25-1.53)‡	1.16 (1.03-1.31)*
NTproBNP§	1.63 (1.49-1.78)‡	—	1.16 (1.04-1.29)†	1.57 (1.44-1.72)‡	1.07 (0.95-1.19)
hs-cTnT§	—	2.45 (2.24-2.70)‡	2.02 (1.80-2.27)‡	—	2.19 (1.94-2.49)‡
Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0)§	—	—	—	2.96 (2.16-4.07)‡	4.46 (3.11-6.40)‡
Cer(d18:1/14:0)§	—	—	—	1.44 (1.01-2.06)*	1.74 (1.17-2.61)†
Cer(d18:1/22:0)§	—	—	—	2.08 (1.32-3.29)†	2.30 (1.37-3.87)†

Data are odds ratio (OR) (95%CI). §Log transformed before analysis.

Model 1. Included traditional risk factors (Hypertension, current smoker, LDL-C, CRP and NTproBNP levels).

Model 2. Only Included hs-cTnT.

Model 3. Included traditional risk factors, and hs-cTnT.

Model 4. Included traditional risk factors, and ceramides.

Model 5. Included traditional risk factors, hs-cTnT and ceramides.

LDL-C, low-density lipoprotein cholesterol; CRP, C-reactive protein; NTproBNP, N-terminal pro-brain natriuretic peptide; hs-cTnT, high-sensitivity cardiac troponin T; Cer, ceramides.

* $P<0.05$.

† $P<0.01$.

‡ $P<0.001$.

Table 5. Discrimination and reclassification of ACS with various multivariable logistic regression models.

Old model	New model	AUC (95% CI)	*P* Value-AUC	NRI (95% CI)	*P* Value-NRI
Traditional factors*	–	0.78 (0.75-0.81)	–	–	–
hs-cTnT	–	0.80 (0.77-0.83)	–	–	–
Traditional factors*	+hs-cTnT	0.81 (0.78-0.84)	0.220	**0.52 (0.40-0.64)**	**<0.001**
Traditional factors*	+Ceramides†	**0.81 (0.78-0.84)**	**<0.001**	**0.35 (0.23-0.47)**	**<0.001**
Traditional factors* + hs-cTnT	+Ceramides†	**0.87 (0.84-0.89)**	**<0.001**	**0.51 (0.39-0.64)**	**<0.001**

*Traditional risk factors include hypertension, current smoker, LDL-C, CRP and NTproBNP levels.
†Ceramides include Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0), Cer(d18:1/14:0) and Cer(d18:1/22:0).
LDL-C, CRP, NT-proBNP, hs-cTnT and ceramides levels are log transformed before analysis.
hs-cTnT, high-sensitivity cardiac troponin T; LDL-C, low-density lipoprotein cholesterol; CRP, C-reactive protein; NT-proBNP, N-terminal pro-brain natriuretic peptide.
P values-AUC and *P* values-NRI are for the difference between old models and new models. Bold used to highlight those *P* values<0.05.

Figure 1: Flow chart.

Figure 2: Receiver operating characteristic (ROC) curves of multi-variable models for the discrimination of ACS.

(A) Traditional risk factors (hypertension, current smoker, LDL-C, CRP and NT-proBNP) and high-sensitive troponin are included in the model. (B) Traditional risk factors (hypertension, current smoker, LDL-C, CRP and NT-proBNP), high-sensitive troponin, Cer(d18:1/14:0), Cer(d18:1/22:0) and Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0) are included in the model.

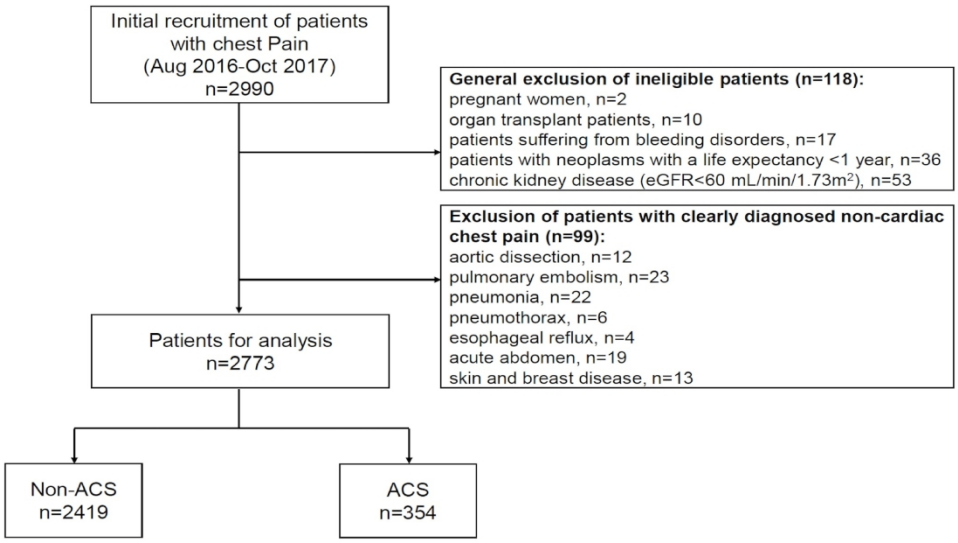

Figure 1: Flow chart.

185x99mm (300 x 300 DPI)

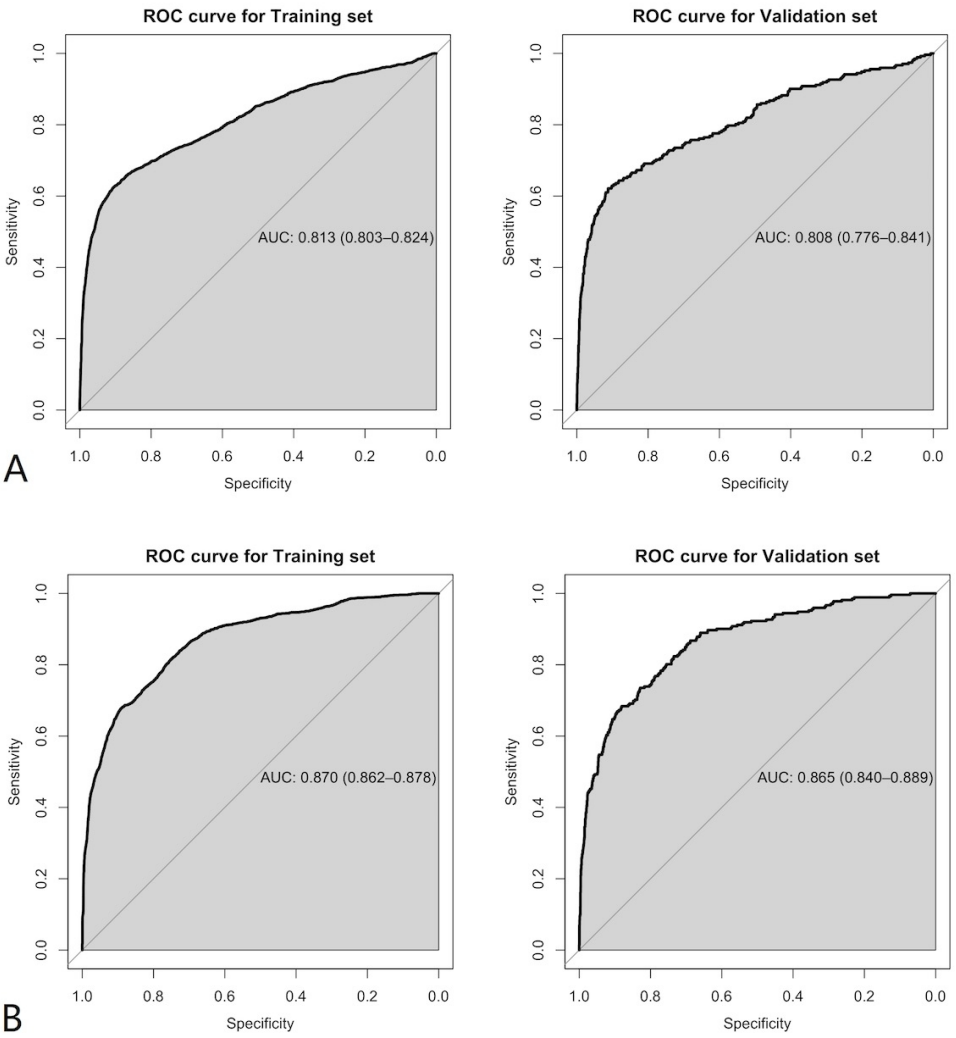

Figure 2: Receiver operating characteristic (ROC) curves of multi-variable models for the discrimination of ACS.

99x110mm (300 x 300 DPI)

Supplementary Table 1 Summary of method information of laboratory analytes.

Analytes	Detection limit	Analytical Range	Reference Interval or Decision Limit	Imprecision Range (Coefficient of Variance, %)
hs-TnT (ng/L)	3	3-10000	<30	<9.4
TG (mmol/L)	0.1	0.1-10.0	<2.26	<2.0
TC (mmol/L)	0.1	0.1-20.7	<5.2	<1.6
HDL-C (mmol/L)	0.08	0.08-3.12	Male >1.45 Female >1.68	<1.5
LDL-C (mmol/L)	0.10	0.10-14.2	<3.34	<1.2
ApoA1 (g/L)	0.002	0.002-2.50	Male 1.10-1.70 Female 1.20-1.90	<3.7
ApoB (g/L)	0.003	0.003-2.50	Male 0.80-1.55 Female 0.75-1.50	<3.5
ApoE (mg/L)	2	2-120	27-45	<50
Lp(a) (mg/L)	30	30-1200	<300	<3.1
NT-proBNP (ng/L)	5	5-35000	<125	<4.2

Supplementary Table 2 Demographics, clinical and lab findings of included patients.

Parameters	Non-ACS	STEMI	NSTEMI	UA	NSTE-ACS
n (%)	2419 (87.2)	124 (4.5)	114 (4.1)	116 (4.2)	230 (8.3)
Age (years)	64.0 (58.0-70.0)	64.0 (55.8-72.0)	63.0 (58.8-71.0)	66.0 (57.0-72.0)	64.0 (58.0-72.0)
Male, n (%)	1634 (68)	98 (82)	89 (79)	66 (58)	155 (69)
Diabetes, n (%)	600 (25)	33 (28)	23 (21)	29 (25)	52 (23)
Hypertension, n (%)	1500 (62)	77 (64)	83 (74)	76 (67)	159 (70)
Current Smoker, n (%)	523 (22)	46 (38)	47 (42)	18 (16)	65 (29)
TG (mmol/L)	1.43 (1.01-2.08)	1.57 (1.15-2.16)	1.60 (1.12-2.04)$^{\Delta\Delta}$	1.75 (1.25-2.60)***	1.68 (1.21-2.28) $^{\#\#}$
TC (mmol/L)	3.74 (3.13-4.49)	4.20 (3.55-4.99)§§	4.28 (3.53-5.25)$^{\Delta\Delta\Delta}$	4.34 (3.47-5.01)***	4.32 (3.51-5.04) $^{\#\#\#}$
HDL-C (mmol/L)	1.090 (0.910-1.310)	0.990 (0.840-1.210)§	0.980 (0.875-1.188)$^{\Delta}$	1.030 (0.905-1.240)	1.010 (0.885-1.230) $^{\#}$
LDL-C (mmol/L)	1.88 (1.36-2.53)	2.36 (1.79-3.12)§§§	2.55 (1.82-3.06)$^{\Delta\Delta\Delta}$	2.23 (1.67-3.03)***	2.47 (1.77-3.05) $^{\#\#\#}$
ApoA1 (g/L)	1.33 (1.17-1.52)	1.19 (1.06-1.38)§§§	1.25 (1.09-1.43)$^{\Delta}$	1.27 (1.13-1.48)	1.25 (1.10-1.46) $^{\#}$
ApoB (g/L)	0.720 (0.570-0.890)	0.815 (0.668-0.992)§	0.915 (0.730-1.062)$^{\Delta\Delta\Delta}$	0.810 (0.650-1.000)**	0.870 (0.670-1.045) $^{\#\#\#}$
ApoE (mg/L)	37.00 (30.00-46.37)	42.00 (34.50-47.25)	42.00 (34.00-51.25)	40.13 (28.00-53.00)	41.00 (31.15-52.00)
Lp(a) (mg/L)	123 (51.00-282.00)	162 (60.50-320.25)	183 (100.75-366.00)	96 (47.00-277.00)	148 (66.00-344.00)
NTproBNP (ng/L)	87.40 (42.33-220.28)	728.20 (259.90-1717.50)§§§	655.50 (171.70-1943.00)$^{\Delta\Delta\Delta}$	105.90 (52.50-215.30)	198.15 (88.35-888.25) $^{\#\#\#}$
CRP (mg/L)	1.500 (0.600-4.800)	8.900 (2.650-20.950)§§§	7.200 (3.100-17.600)$^{\Delta\Delta\Delta}$	2.50 (1.00-5.40)	4.55 (1.40-10.20) $^{\#\#\#}$
hs-cTnT (ng/L)	8 (5-13)	205 (30-883)§§§	162 (74-624)$^{\Delta\Delta\Delta}$	8 (5-11)	49 (9-263) $^{\#\#\#}$

STEMI vs. non-ACS: $^{\S}p<0.05$, $^{\S\S}p<0.01$, $^{\S\S\S}p<0.001$. NSTEMI vs. non-ACS: $^{\Delta}p<0.05$, $^{\Delta\Delta}p<0.01$, $^{\Delta\Delta\Delta}p<0.001$

UA vs. non-ACS: *$p<0.05$, **$p<0.01$, ***$p<0.001$. NSTE-ACS vs. non-ACS, $^{\#}p<0.05$ $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$

ACS, acute coronary syndrome; STEMI, ST-elevation myocardial infarction; NSTEMI, non-ST-elevation myocardial infarction; UA, unstable angina; NSTE-ACS, non-ST-elevation acute coronary syndrome.

Supplementary Table 3 Ceramides levels of included patients.

Parameters	Non-ACS	STEMI	NSTEMI	UA	NSTE-ACS
Cer(d18:1/16:0)	0.198 (0.162-0.241)	0.243 (0.206-0.282)§§§	0.228 (0.200-0.286)^{ΔΔΔ}	0.242 (0.203-0.285)***	0.235 (0.201-0.286) $^{###}$
Cer(d18:1/18:0)	0.049 (0.036-0.065)	0.069 (0.052-0.086)§§§	0.064 (0.047-0.085)^{ΔΔΔ}	0.068 (0.052-0.080)***	0.066 (0.050-0.083) $^{###}$
Cer(d18:1/24:0)	2.053 (1.592-2.672)	2.134 (1.675-2.702)	2.152 (1.674-2.737)	2.218 (1.684-2.771)	2.183 (1.675-2.756)
Cer(d18:1/24:1(15Z))	0.569 (0.436-0.739)	0.706 (0.571-0.980)§§§	0.669 (0.519-0.906)^{ΔΔΔ}	0.764 (0.593-1.061)***	0.732 (0.552-0.950) $^{###}$
Cer(d18:1/14:0)	0.003 (0.002-0.004)	0.003 (0.002-0.004)§	0.003 (0.002-0.004)^{ΔΔΔ}	0.003 (0.003-0.004)***	0.003 (0.002-0.004) $^{###}$
Cer(d18:1/20:0)	0.051 (0.039-0.065)	0.065 (0.051-0.081)§§§	0.061 (0.049-0.076)^{ΔΔΔ}	0.063 (0.054-0.082)***	0.062 (0.052-0.078) $^{###}$
Cer(d18:1/22:0)	0.375 (0.294-0.480)	0.417 (0.334-0.518)	0.403 (0.324-0.520)^{ΔΔΔ}	0.439 (0.350-0.594)***	0.413 (0.340-0.550) $^{###}$
Cer(d18:0/16:0)	0.010 (0.007-0.013)	0.010 (0.008-0.013)	0.011 (0.008-0.015)^{ΔΔΔ}	0.013 (0.010-0.017)***	0.012 (0.009-0.016) $^{###}$
Cer(d18:0/18:0)	0.005 (0.003-0.007)	0.007 (0.004-0.011)§§§	0.007 (0.004-0.011)^{ΔΔΔ}	0.007 (0.005-0.011)***	0.007 (0.004-0.011) $^{###}$
Cer(d18:0/24:0)	0.060 (0.042-0.086)	0.063 (0.043-0.085)	0.066 (0.049-0.087)^{ΔΔ}	0.075 (0.055-0.104)**	0.071 (0.052-0.096) $^{##}$
Cer(d18:0/24:1(15Z))	0.028 (0.019-0.041)	0.037 (0.025-0.053)§§§	0.039 (0.024-0.054)^{ΔΔΔ}	0.045 (0.032-0.065)***	0.042 (0.028-0.06) $^{###}$
Cer(d18:1/24:1)	0.258 (0.192-0.372)	0.277 (0.204-0.377)	0.300 (0.230-0.366)^{ΔΔΔ}	0.333 (0.228-0.413)**	0.313 (0.229-0.389) $^{###}$
Cer(d18:1/16:0)/Cer(d18:1/24:0)	0.095 (0.079-0.114)	0.111 (0.091-0.132)§§§	0.103 (0.088-0.131)^{ΔΔΔ}	0.109 (0.090-0.133)***	0.107 (0.090-0.133) $^{###}$
Cer(d18:1/18:0)/Cer(d18:1/24:0)	0.023 (0.018-0.031)	0.032 (0.023-0.040)§§§	0.030 (0.023-0.040)^{ΔΔΔ}	0.031 (0.023-0.039)***	0.031 (0.023-0.039) $^{###}$
Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0)	0.261 (0.211-0.345)	0.312 (0.253-0.414)§§§	0.314 (0.234-0.411)^{ΔΔΔ}	0.353 (0.267-0.481)***	0.327 (0.255-0.429) $^{###}$

STEMI vs. non-ACS: $^{§}p<0.05$, $^{§§}p<0.01$, $^{§§§}p<0.001$. NSTEMI vs. non-ACS: $^{Δ}p<0.05$, $^{ΔΔ}p<0.01$, $^{ΔΔΔ}p<0.001$

UA vs. non-ACS: *$p<0.05$, **$p<0.01$, ***$p<0.001$. NSTE-ACS vs. non-ACS, $^{#}p<0.05$ $^{##}p<0.01$, $^{###}p<0.001$

ACS, acute coronary syndrome; STEMI, ST-elevation myocardial infarction; NSTEMI, non-ST-elevation myocardial infarction; UA, unstable angina; NSTE-ACS, non-ST-elevation acute coronary syndrome.

Supplementary Table 4. Correlations of ceramides levels and ratios with demographics and conventional ACS biomarkers.

Ceramides	Age	TG	TC	HDL-C	LDL-C	ApoA1	ApoB	ApoE	Lp(a)	hs-cTnT	NTproBNP	CRP
Cer(d18:1/16:0)	**0.044**	**0.262**	**0.515**	0.015	**0.466**	**0.069**	**0.453**	**0.333**	**0.068**	**0.124**	**0.155**	**0.231**
Cer(d18:1/18:0)	-0.021	**0.305**	**0.331**	**-0.074**	**0.278**	-0.006	**0.318**	**0.268**	**0.077**	**0.108**	**0.121**	**0.228**
Cer(d18:1/24:0)	**-0.077**	**0.397**	**0.491**	**-0.063**	**0.414**	**0.066**	**0.459**	**0.363**	0.002	-0.024	**-0.059**	**0.109**
Cer(d18:1/24:1(15Z))	0.012	**0.268**	**0.347**	-0.021	**0.292**	0.025	**0.317**	**0.271**	**0.083**	**0.095**	**0.142**	**0.181**
Cer(d18:1/14:0)	0.009	**0.166**	**0.428**	**0.168**	**0.362**	**0.208**	**0.328**	**0.271**	0.007	**-0.069**	-0.008	**0.082**
Cer(d18:1/20:0)	-0.021	**0.309**	**0.343**	**-0.048**	**0.277**	0.021	**0.312**	**0.264**	**0.080**	**0.095**	**0.114**	**0.174**
Cer(d18:1/22:0)	**-0.083**	**0.405**	**0.480**	**-0.070**	**0.401**	**0.076**	**0.478**	**0.394**	0.013	-0.011	-0.015	**0.128**
Cer(d18:0/16:0)	**0.051**	**0.228**	**0.396**	0.013	**0.342**	**0.070**	**0.341**	**0.297**	0.022	**0.054**	**0.085**	**0.168**
Cer(d18:0/18:0)	0.011	**0.245**	**0.275**	**-0.088**	**0.246**	-0.019	**0.274**	**0.236**	0.027	**0.106**	**0.093**	**0.231**
Cer(d18:0/24:0)	-0.004	**0.204**	**0.389**	0.028	**0.352**	**0.068**	**0.305**	**0.232**	0.010	0.025	0.011	**0.130**
Cer(d18:0/24:1(15Z))	0.016	**0.238**	**0.308**	-0.033	**0.271**	0.023	**0.281**	**0.244**	**0.046**	**0.091**	**0.102**	**0.195**
Cer(d18:1/24:1)	0.034	**-0.088**	**0.257**	**0.164**	**0.270**	**0.106**	**0.210**	**0.092**	**0.053**	**0.051**	**0.093**	**0.061**
Cer(d18:1/16:0)/Cer(d18:1/24:0)	**0.153**	**-0.240**	**-0.091**	**0.098**	**-0.044**	-0.018	**-0.114**	**-0.122**	**0.067**	**0.148**	**0.236**	**0.097**
Cer(d18:1/18:0)/Cer(d18:1/24:0)	**0.049**	-0.031	**-0.077**	-0.023	**-0.065**	**-0.064**	**-0.061**	-0.037	**0.074**	**0.136**	**0.175**	**0.144**
Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0)	**0.078**	**-0.095**	**-0.103**	0.022	**-0.084**	**-0.050**	**-0.094**	**-0.063**	**0.090**	**0.120**	**0.190**	**0.086**

Data are r values between ceramides levels and ratios with demographic parameters and conventional ACS biomarkers. P<0.05 in bold.

TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; ApoE, apolipoprotein E; LP(a), lipoprotein(a); NTproBNP, N-terminal pro-brain natriuretic peptide; CRP, C-reactive protein; hs-cTnT, high-sensitivity cardiac troponin T; Cer, ceramides.

Sample size

Conventional sample size calculations based on the effect size and variation of data are generally not applicable for observational studies especially involving complex multivariate analyses. Instead, we use the concept of margin of error to give a general indication of the precision of our estimates. The margin of error for a particular statistic of interest is usually defined as the radius (or half the width) of the confidence interval for that statistic. A sample size of 2773 patients in our study will give a margin of error of 1.8%, at a 95% confidence level, which indicates a high level of accuracy in our results.

Section & Topic	No	Item	Reported on page #
TITLE OR ABSTRACT			
	1	Identification as a study of diagnostic accuracy using at least one measure of accuracy (such as sensitivity, specificity, predictive values, or AUC)	1
ABSTRACT			
	2	Structured summary of study design, methods, results, and conclusions (for specific guidance, see STARD for Abstracts)	3
INTRODUCTION			
	3	Scientific and clinical background, including the intended use and clinical role of the index test	6-7
	4	Study objectives and hypotheses	7
METHODS			
Study design	**5**	Whether data collection was planned before the index test and reference standard were performed (prospective study) or after (retrospective study)	9
Participants	**6**	Eligibility criteria	7
	7	On what basis potentially eligible participants were identified (such as symptoms, results from previous tests, inclusion in registry)	7
	8	Where and when potentially eligible participants were identified (setting, location and dates)	7
	9	Whether participants formed a consecutive, random or convenience series	7
Test methods	**10a**	Index test, in sufficient detail to allow replication	9
	10b	Reference standard, in sufficient detail to allow replication	8
	11	Rationale for choosing the reference standard (if alternatives exist)	NA
	12a	Definition of and rationale for test positivity cut-offs or result categories of the index test, distinguishing pre-specified from exploratory	NA
	12b	Definition of and rationale for test positivity cut-offs or result categories of the reference standard, distinguishing pre-specified from exploratory	8
	13a	Whether clinical information and reference standard results were available to the performers/readers of the index test	8
	13b	Whether clinical information and index test results were available to the assessors of the reference standard	9
Analysis	**14**	Methods for estimating or comparing measures of diagnostic accuracy	10
	15	How indeterminate index test or reference standard results were handled	NA
	16	How missing data on the index test and reference standard were handled	NA
	17	Any analyses of variability in diagnostic accuracy, distinguishing pre-specified from exploratory	NA
	18	Intended sample size and how it was determined	supplementary information
RESULTS			
Participants	**19**	Flow of participants, using a diagram	Figure 1
	20	Baseline demographic and clinical characteristics of participants	11 & Table 1
	21a	Distribution of severity of disease in those with the target condition	Table1 & Supplementary table 2
	21b	Distribution of alternative diagnoses in those without the target condition	Table 1
	22	Time interval and any clinical interventions between index test and reference standard	NA
Test results	**23**	Cross tabulation of the index test results (or their distribution) by the results of the reference standard	NA
	24	Estimates of diagnostic accuracy and their precision (such as 95% confidence intervals)	12-13
	25	Any adverse events from performing the index test or the reference standard	NA
DISCUSSION			
	26	Study limitations, including sources of potential bias, statistical uncertainty, and generalisability	14
	27	Implications for practice, including the intended use and clinical role of the index test	13
OTHER INFORMATION			

	28	Registration number and name of registry	NA
	29	Where the full study protocol can be accessed	8
	30	Sources of funding and other support; role of funders	4

STARD 2015

AIM

STARD stands for "Standards for Reporting Diagnostic accuracy studies". This list of items was developed to contribute to the completeness and transparency of reporting of diagnostic accuracy studies. Authors can use the list to write informative study reports. Editors and peer-reviewers can use it to evaluate whether the information has been included in manuscripts submitted for publication.

EXPLANATION

A **diagnostic accuracy study** evaluates the ability of one or more medical tests to correctly classify study participants as having a **target condition.** This can be a disease, a disease stage, response or benefit from therapy, or an event or condition in the future. A medical test can be an imaging procedure, a laboratory test, elements from history and physical examination, a combination of these, or any other method for collecting information about the current health status of a patient.

The test whose accuracy is evaluated is called **index test.** A study can evaluate the accuracy of one or more index tests. Evaluating the ability of a medical test to correctly classify patients is typically done by comparing the distribution of the index test results with those of the **reference standard**. The reference standard is the best available method for establishing the presence or absence of the target condition. An accuracy study can rely on one or more reference standards.

If test results are categorized as either positive or negative, the cross tabulation of the index test results against those of the reference standard can be used to estimate the **sensitivity** of the index test (the proportion of participants *with* the target condition who have a positive index test), and its **specificity** (the proportion *without* the target condition who have a negative index test). From this cross tabulation (sometimes referred to as the contingency or "2x2" table), several other accuracy statistics can be estimated, such as the positive and negative **predictive values** of the test. Confidence intervals around estimates of accuracy can then be calculated to quantify the statistical **precision** of the measurements.

If the index test results can take more than two values, categorization of test results as positive or negative requires a **test positivity cut-off**. When multiple such cut-offs can be defined, authors can report a receiver operating characteristic (ROC) curve which graphically represents the combination of sensitivity and specificity for each possible test positivity cut-off. The **area under the ROC curve** informs in a single numerical value about the overall diagnostic accuracy of the index test.

The **intended use** of a medical test can be diagnosis, screening, staging, monitoring, surveillance, prediction or prognosis. The **clinical role** of a test explains its position relative to existing tests in the clinical pathway. A replacement test, for example, replaces an existing test. A triage test is used before an existing test; an add-on test is used after an existing test.

Besides diagnostic accuracy, several other outcomes and statistics may be relevant in the evaluation of medical tests. Medical tests can also be used to classify patients for purposes other than diagnosis, such as staging or prognosis. The STARD list was not explicitly developed for these other outcomes, statistics, and study types, although most STARD items would still apply.

DEVELOPMENT

This STARD list was released in 2015. The 30 items were identified by an international expert group of methodologists, researchers, and editors. The guiding principle in the development of STARD was to select items that, when reported, would help readers to judge the potential for bias in the study, to appraise the applicability of the study findings and the validity of conclusions and recommendations. The list represents an update of the first version, which was published in 2003.

More information can be found on http://www.equator-network.org/reporting-guidelines/stard.

The Effect of Combined Testing of Ceramides with High-sensitive Troponin T on the Detection of Acute Coronary Syndrome in Patients with Chest Pain in China： a prospective observational study

Authors: Kang Yao[1*], MD; Yanzhong Wang[2*], PhD; Xuebo Liu[3], MD; Cheng-Xing Shen[4], MD; Wei Hu[5], MD; Zhe Wang[1]; Runda Wu[1], MD; Xianglin Tang[1], MD; Aijun Sun[1,6], MD; Yunzeng Zou[1], MD; Juying Qian[1], MD; Guangyu Wu[7], MD, PhD; Xin Guo[7], MD; Xiaoliang Cheng[7], PhD; Junbo Ge[1], MD

[*] Drs Yao and Wang contributed equally.

1. Department of Cardiology, Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Fudan University, Shanghai, China
2. School of Population Health and Environmental Sciences, King's College London, London, UK
3. Department of Cardiology, Tongji Hospital, Tongji University, Shanghai, China
4. Department of Cardiology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China
5. Department of Cardiology, Minhang Hospital, Fudan University, Shanghai, China
6. Institute of Biomedical Science, Fudan University, Shanghai, China
7. Qlife Lab Co., Ltd., 700 Yishan Road, Shanghai Putian Information Industrial Park, Shanghai, China

Corresponding author:

Prof. Junbo Ge, MD, FACC, FESC

Department of Cardiology, Shanghai Institute of Cardiovascular Diseases,

Zhongshan Hospital, Fudan University

180 Fenglin Road, Shanghai, China. Postcode: 200032

Tel: 0086 (0)21 64041990-2152; Fax: 0086 (0)21 64437078

Email: ge.junbo2@zs-hospital.sh.cn

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Abstract

Objective Ceramides are associated with coronary plaque vulnerability. We aim to investigate the potential diagnostic value of ceramides for Acute Coronary Syndrome (ACS) in Chinese patients with chest pain.

Design Prospective observational survey.

Setting Shanghai, China, 2016-7.

Participants 2773 patients with chest pain from four hospitals in Shanghai, China between August 2016 and October 2017.

Main outcome measures Performance of metabolites model in detection of ACS cases including ST-elevation myocardial infarction (STEMI), non-ST-elevation myocardial infarction (NSTEMI), and unstable angina (UA).

Results – Plasma levels of 12 ceramides molecules and corresponding ratios were compared between patients diagnosed with ACS and those without. Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0) ratio, Cer(d18:1/14:0) and Cer(d18:1/22:0) were independent predictors of ACS after adjustment of traditional risk factors and high sensitive-cTnT. Receiver operating characteristic curve (ROC) analysis showed a significant improvement in detecting ACS in the multi-variable model with ceramides as compared to that without [0.865 (0.840-0.889) *vs.* 0.808 (0.776-0.841), $P < 0.001$].

Conclusion – Distinct plasma ceramides are independent diagnostic predictors of ACS among patients with chest pain. Ceramides together with high-sensitive troponin and traditional factors showed great potential in identifying ACS among patients with chest pain.

Article Summary

Strengths and limitations of this study

- This is the first study with systematic demonstration of plasma levels of 12 ceramide molecules in 2773 Chinese patients with chest pain.
- Performance of ceramides levels in diagnosing acute coronary syndrome was assessed and validated in multi-center clinical studies.
- The time of ceramides level elevation after ACS occurrence cannot be assessed in the study design.

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Author Contributions: Study concept and design (GJ, YK, WY); recruitment of patients and acquisition of samples (YK, LX, SC, HW, WZ, WR, TX, SA, ZY, QJ); analysis and interpretation of data (WY, WG, GX, CX); drafting first version of the manuscript (WG, WY); critical revision of the manuscript and approval of final version (all authors).

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Conflict of interest: Q-life lab holds patents for the diagnostic use of ceramides for cardiovascular risk determination in China. WG, GX and CX work for Q-life and GX and CX are also shareholders. Other authors declare no conflict of interest.

Patient consent: Obtained.

Ethics approval: This study was approved by the Ethics Committee of Zhongshan Hospital, the Ethics Committee of Tongji Hospital, the Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital, and the Ethics Committee of Minhang Hospital.

Data sharing statement: Data is available with the corresponding author and will be available on request.

Patient and Public Involvement

This study was conducted without patient and public involvement. The patients were not invited to comment on the study design and were not consulted to develop outcomes or interpret the results. Patients were not invited to contribute to the writing or editing of this document for readability or accuracy.

The study complies with the Declaration of Helsinki and was approved by the Ethics Committees of the participating clinical centers. Written informed consent was obtained from all participants. Participation was voluntary and could be terminated at any time. All data were used strictly confidentially and anonymously. There are no plans to disseminate the results of the research to study participants.

1. Introduction

Acute Coronary Syndrome (ACS), which includes both myocardial infarction (MI) and unstable angina (UA), is estimated to affect 1.4 million people in the United States, and 2.5 million people in China per year.[1 2] However, patients with acute chest pain suggestive of ACS present with a heterogeneous array of conditions, including both non-ischaemic and ischemic chest pain.[3] Only 17% of these are finally diagnosed as ACS caused by atherosclerotic and ischaemic heart disease.[3]

The likelihood of ACS in patients with chest pain is estimated via the entire clinical picture, including symptoms and physical examination findings, disease history, electrocardiogram (ECG) changes, and biomarkers results. Among biomarkers, cardiac troponins play a central role in establishing a diagnosis and stratifying risk. Troponins are more specific and sensitive than the traditional cardiac enzymes such as creatine kinase (CK), its isoenzyme MB (CK-MB), and myoglobin.[4] However, there has been an increasing recognition of other biomarkers recently.[5] Considerable effort has been made to improve the multi-biomarker based evaluation and management of ACS. Altered lipid metabolism associated with inflammation and oxidative stress initiate the pathological changes, including the formation of lipid-laden foam cells and the necrotic lipid core of unstable plaque.[6 7] As many lipid types are essential in atherogenesis, they should be used for ACS prediction.[6]

Ceramides are a family of lipid molecules that are found in high concentrations within cell membranes and play a key role in a variety of physiology functions including apoptosis, cell growth, cell adhesion and plasma membrane integrity maintenance.[8] All tissues can synthesize ceramides de novo from saturated fats and sphingosine.[9] However, lesional low density lipoprotein (LDL) is known to be rich in ceramide in the atherosclerotic plaque, and it contains 10- to 50-fold-higher content of ceramide when compared with plasma LDL.[10] An arterial-wall sphingomyelinase (SMase) hydrolyzes the sphingomyelin (SM) of retained LDL.[10] Plasma ceramide (d18:1/16:0), is associated with the fraction of necrotic core tissue and lipid core burden in coronary atherosclerosis.[11] Inflammatory cytokine tumor necrosis factor alpha was shown to induce the production of ceramide in vascular endothelial cells,[12 13]

via the activation of SMase, accompanied by reactive oxygen species production.[14] High content of oxidized LDL (ox-LDL) in the plaque is capable of activating precursors to matrix metallopeptidase 2, propagating the signalling of the ceramide pathway by activation of SMase.[15] These findings suggest abnormal regulation of ceramides might be related to plaque instability and triggering of ACS.

Previous clinical research found that circulating ceramides are elevated in patients with hypertension,[16] obesity,[17] [18] and type 2 diabetes mellitus.[18] Ceramides ratio levels such as Cer(d18:1/16:0) / Cer(d18:1/24:0) ratio, Cer(d18:1/18:0) / Cer(d18:1/24:0) ratio, and Cer(d18:1/24:1) / Cer(d18:1/24:0) ratio were proposed as useful biomarkers for cardiovascular death (CV death) prediction, as assessed by three independent coronary artery disease (CAD) cohorts.[19]

Although several studies have shown plasma levels of specific ceramide molecules are correlated with diagnosis of patients with cardiac events, no studies to date has evaluated the role of these ceramide molecules in detecting ACS from patients with chest pain. In this study, we aim to assess the value of ceramides in detection of ACS in patients with chest pain and whether a combination of ceramides and troponin could improve the diagnostic power.

2. Methods

2.1. Study design and participants

This is a prospective observational study involving four University affiliated hospitals in Shanghai, China. A total of 2990 patients with chest pain were consecutively recruited in chest pain outpatient during Monday to Friday between August 2016 and October 2017, and 2773 patients were finally admitted to wards of cardiology after pre-screening by the exclusion criteria. Sample size calculation is shown in Supplementary File. Patients were eligible if they were aged ≥18 and ≤ 80 years, presented with signs and symptoms of chest pain and agreed to participate in this study. The exclusion criteria were pregnant women; organ transplant patients; patients suffering from bleeding disorders; patients

with neoplasms with a life expectancy <1 year; chronic kidney disease (eGFR ＜60 mL/min/1.73m^2) and patients with a clear non-cardiac chest pain. A flow chart describing the inclusion and exclusion process is shown as Figure 1. Full study protocol is provided upon request to the corresponding author. The study complies with the Declaration of Helsinki and was approved by the Ethics Committee of Zhongshan Hospital, the Ethics Committee of Tongji Hospital, the Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital, and the Ethics Committee of Minhang Hospital. Written informed consent was obtained from all participants.

2.2. Clinical and laboratory data

Clinical history, physical examination findings and blood tests including renal and liver functions, C-reactive protein (CRP), D-dimer and high-sensitive cTnT (Roche) levels, ECG, coronary angiogram and echocardiogram findings were recorded in all patients. Troponin levels were measured by electrochemiluminescence method using high sensitive-cTnT assay (Roche Diagnostics) on Roche Cobas e601. The coefficient of variation in the hs-cTnT assay is ⩽10% at the cut-off value of 13 ng/L. The 99 percentile upper reference limit of hs-cTnT assay is 14 ng/L. Besides, the assay also has a limit of blank of 3 ng/L and a limit of detection of 5 ng/L, and the analytical range is 3-10000 ng/L. Regular blood lipid tests were conducted using standard methods. Specifically, total cholesterol (TC) was measured by enzymatic cholesterol method using cholesterol oxidase/peroxidase aminophenazone (COD-PAP) reagent while total triglyceride (TG) was measured by Glycerol-3-Phosphate oxidase/peroxidase anti-peroxidase method (GPO-PAP) method. Low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were measured by homogenous PEG modification enzyme method. Other blood biomedical assay of apolipoprotein (AI, AII, B, and E), lipoprotein (a), and lipoprotein-associated phospholipase were performed by immune transmission turbidity method. Performers of the above tests were blinded to the diagnosis of diseases. The detection limit, analytical range, and reference interval or decision limit are summarized in Supplementary Table 1.

2.3. Diagnosis of ACS

The diagnosis of ACS including UA, Non-ST segment elevation myocardial infarction (NSTEMI), and ST-segment elevation myocardial infarction (STEMI) was made by two independent cardiologists by reviewing all patients' notes, including symptoms, 12-lead ECG, and blood tests results according to the 2014 American Heart Association /American College of Cardiology guidelines and international definition of myocardial infarction version 3. Type 1 and type 2 MI were both included.[20 21] The agreement level and kappa value between the 2 cardiologists were 0.99 and 0.98. The cases where the 2 cardiologists disagreed were reviewed by a senior cardiologist. All the cardiologists were blinded to ceramide levels. Cardiac chest pain with persistent ECG findings of elevation in the ST segment indicate STEMI. Cardiac chest pain and elevations in troponins levels without ST elevation indicate NSTEMI. Those with presence of 1 or more of 3 principal ischemic symptoms ((1) rest angina (lasting >20 minutes), (2) new-onset (<2 months previously) severe angina, and (3) a crescendo pattern of occurrence (increasing in intensity, duration, frequency, or any combination of these factors)) without elevations in cardiac troponins are defined as UA.[5] Participants were diagnosed with ACS when there is evidence of UA, NSTEMI or STEMI.

2.4. Quantification of plasma ceramides

Potential ceramides were generated from previous untargeted and targeted ceramide profile studies.[22 23] Analyses of 12 plasma ceramides were performed using a Waters Xevo TQ-S mass spectrometer (Manchester, UK) equipped with Waters Acuity UPLC I-Class (Milford, MA, USA) in a hospital and Qlife lab collaborative laboratory based in Department of Cardiology. The mass spectrometry was operated in multiple-reaction monitoring (MRM) mode with ESI-positive ionization. The capillary voltage was set at 3.0 kV. and the source temperature was 120℃. The desolvation temperature and gas flow were 400℃ and 800L/h, respectively. The source offset was maintained at 60V.

Blood samples for ceramides test were collected and centrifuged using ethylene diamine tetraacetic acid (EDTA) anticoagulation tube at admission. Plasma were immediately stored in -80℃ for future analysis. Ceramides test were taken immediately after samples of all patients were collected. Before analysis, the samples were thawed at room temperature, then a volume of 800 μl of protein precipitation solution (isopropanol) that containing D7-Cer d18:1/16:0 (0.01 pmol/μl), D7-Cer d18:1/18:0 (0.005 pmol/μl), D7-Cer d18:1/24:0 (0.015 pmol/μl) and D7-Cer d18:1/24:1 (0.015 pmol/μl) was pipetted into 1.5 mL Eppendorff tube after addition of 50 μL of plasma sample. The mixture was thoroughly vortexed for 10 min followed by 5-min centrifugation at 14,000 rpm. Supernatant (70 μL) was collected for LC/MS/MS analysis. Ceramides ratios were calculated using Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/24:1(15Z)) and Cer(d18:1/24:0).[23] Lab technicians who were in charge for ceramides test were blinded to disease diagnosis.

2.5. Statistical methods

Continuous variables are summarized as mean (standard deviation) or median (interquartile range) and categorical data as count (percentage). Student's t-test, Mann-Whitney U, and Wilcoxon signed rank tests were used to test differences in continuous variables where appropriate, and Chi-squared test was used for proportions. Univariate logistic regression was used to analyse the association of each variable and binary outcome. Multivariable logistic regression analyses were undertaken to identify the demographic, clinical and laboratory factors associated with ACS in patients with chest pain after adjusting for potential confounders and the final models included variables significantly associated with ACS.

Training and validation cohorts were developed via a 10-fold cross-validation approach repeated 10 times.[24] The estimate of prediction error is almost unbiased under 10-fold cross-validation. Repeated 10 times that minimizes the cross-validated error is then used to build the final model.[25] [26] The performance and discrimination ability of the five models were assessed using the area under the

receiver operating curve (AUROC) and net reclassification index (NRI). ROC curves and NRI values were compared among models in training and validation datasets.

All tests were two-tailed, and *P* values less than 0.05 were considered statistically significant. Odds ratios (OR) with 95% confidence intervals (CIs) for the chosen clinical and laboratory variables in the logistic regression models and AUROC and NRI values with 95% CIs for different test datasets were calculated. Statistical analyses were done with statistical software R, version 3.4.3.

2.6.Patient and Public Involvement

This study was conducted without patient and public involvement.

3. Results

3.1. Demographics, lipid profiles and laboratory findings of patients

The demographics, clinical and lab findings of all participants are reported in Table 1. Serial troponin levels were measured at 0h-3h after admission in all recruited patients and all patients underwent echocardiography and coronary angiography. In 2773 patients with chest pain, 354 (12.8%) were diagnosed with ACS, among whom 116 (4.2%) were UA, 114 (4.1%) were NSTEMI, and 124 (4.5%) were STEMI. All 116 patients with UA had a significant stenosis on coronary angiography. Compared to those without ACS, patients with ACS are more likely to be men (73.44% vs 67.57%), to be current smokers (31.35% vs 21.62%), and to have hypertension (68.92% vs 62.00%). Lipid profiles and lipoproteins including TG, TC, LDL-C, ApoB, ApoE, Lipoprotein(a) (Lp(a)) were significantly elevated in patients diagnosed with ACS while HDL-C and apolipoprotein A1 (ApoA1) were significantly lower in ACS patients (all $p<0.001$, except for Lp(a), $p=0.023$). CRP and cardiac damage markers including hs-cTnT and N-terminal pro-brain natriuretic peptide (NTproBNP) levels were significantly higher in ACS patients (all $p<0.001$). Demographics, lipid profiles and laboratory findings of patients diagnosed with STEMI, NSTEMI and UA were also reported in Supplementary Table 2.

3.2. Plasma ceramides levels in ACS patients

Table 2 presents the levels of twelve ceramides molecules in patients with and without ACS. The levels of 12 ceramide molecules (Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/24:0), Cer(d18:1/24:1(15Z)), Cer(d18:1/14:0), Cer(d18:1/20:0), Cer(d18:1/22:0), Cer(d18:0/16:0), Cer(d18:0/18:0), Cer(d18:0/24:0), Cer(d18:0/24:1(15Z)), Cer(d18:1/24:1)) were all significantly elevated in patients with ACS compared to those without (all $p<0.001$, except for Cer(d18:1/24:0), $p=0.028$) (Table 2). In addition, 3 ceramide ratios (Cer(d18:1/16:0)/Cer(d18:1/24:0), Cer(d18:1/18:0)/Cer(d18:1/24:0) and Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0)) were also significantly increased in ACS patients with all p values less than 0.001. In patients diagnosed with UA and NSTEMI, levels of ceramides and ceramide ratios were also significantly higher compared to patients diagnosed with non-ACS (Supplementary Table 3).

3.3. Association between plasma ceramides and traditional and cardiac damage markers

The associations between plasma ceramides with traditional ACS risk factors and cardiac markers for myocardial injury are shown in Supplementary Table 4. Most of the 12 ceramide molecules and ceramide ratios were moderately correlated with lipid profiles including TG, TC, LDL-C, ApoB and ApoE, while CRP was shown to be weakly correlated with ceramides. There was no significant correlation between ceramides and myocardial damage markers including hs-cTnT and NTproBNP.

3.4. Clinical predictors and multi-variable models for ACS

Clinical predictors for ACS were identified in univariate logistic regression analysis and are shown in Table 3. Being male, having hypertension, and being current smoker were significantly associated with ACS ($p<0.05$). Besides, most of the lipid profile (TG, TC, HDL-C, LDL-C, ApoA1 and ApoB) were significantly associated with ACS (all $p<0.001$). Also, increased levels of hs-cTnT (OR and 95%CI: 2.45 [2.23-2.70]), NTproBNP (1.73 [1.60-1.87]) and CRP (1.73 [1.58, 1.90]) were significantly

associated with ACS. Furthermore, all 12 ceramides levels and 3 ceramides ratios were significantly associated with ACS with all p<0.001 except Cer(d18:1/24:0) with p=0.025 (Table 3).

Multivariable logistic regression analyzing independently significant predictors of ACS are shown in Table 4. Model 1 included traditional risk factors such as hypertension, current smoker, CRP, LDL-C and NTproBNP (all p<0.001 except for hypertension p=0.033). Model 2 only included hs-cTnT (2.454 [2.239-2.701], p<0.001). Model 3 included traditional risk factors and hs-cTnT. Current smoker, CRP, LDL-C, NTproBNP as well as hs-cTnT were independently significant in detecting patients with ACS. Model 4 included traditional risk factors and ceramides levels, with OR (95%CI) of Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0): 2.960 (2.156-4.072), Cer(d18:1/14:0): 1.439 (1.007-2.062) and Cer(d18:1/22:0): 2.079 (1.315-3.288). Model 5 included traditional risk factors, hs-cTnT (2.190, [1.939-2.485]) and ceramides levels (Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0): 4.455 [3.112-6.396], Cer(d18:1/14:0): 1.73 [1.166-2.605], and Cer(d18:1/22:0): 2.301 [1.371, 3.866], all p<0.01). Ceramides were shown to be independently associated with ACS after adjusting for traditional factors and hs-cTnT.

3.5. Discrimination and reclassification of ACS with various multi-variable logistic regression models

As shown in Table 5, the model including traditional factors (hypertension, current smoker, LDL-C, CRP and NT-proBNP level) had an area under the curve (AUC) of 0.780 (0.750-0.811) in diagnosing ACS. AUC of hs-cTnT in predicting ACS was 0.800 (0.767-0.833). After combining traditional factors and hs-cTnT, the AUC became 0.808 (0.776-0.841) (Figure 2A). The AUC of model including traditional factors and ceramides was 0.811 (0.784-0.837). Notably, when combining traditional factors, hs-cTnT, and ceramides, the AUC of the model rose to 0.865 (0.840-0.889) (Figure 2B). We also compared discrimination and reclassification power of these models. Results showed that ceramides significantly improved the AUC of the model with traditional factors and hs-cTnT (0.865 [0.840-0.889] *vs.* 0.808[0.776-0.841], p<0.001). Net reclassification index (NRI) analyses also showed that the model

including traditional factors and hs-cTnT was significantly improved by the inclusion of ceramides (NRI=0.511 (0.388-0.635), p<0.001).

4. Discussion

In this study of 2773 Chinese patients with chest pain from four hospitals in Shanghai, China, levels of 12 ceramides were systematically evaluated together with that of traditional risk factors and high-sensitive troponin. Ceramides and their ratios were shown to be independent predictors of ACS in patients with chest pain after adjustment for traditional risk factors (e.g., age, sex, body mass index (BMI), smoking status, and blood cholesterol) and cardiac damage biomarkers (i.e., high-sensitive troponin). The proposed model with ceramides showed substantial promise and improved value as a risk evaluation tool for ACS with improved performance on model discrimination and reclassification. With targeted liquid chromatography–mass spectrometry based lipidomic approach, we have successfully established the characteristics of cardiac risk-related ceramides from 2773 Chinese patients with chest pain and confirmed our hypothesis that plasma levels of ceramide subspecies correlate with atherosclerotic plaque instability and hence might be used as lipidomic markers for ACS.

This is to our knowledge the first study with systematic demonstration of plasma levels of 12 ceramide molecules in a Chinese population. Also, the selection of two ceramides molecules and one ceramide ratio as potential biomarkers for ACS has not been reported before. Although our ceramides-based diagnostic model showed great potential in identifying ACS among patients with chest pain, its clinical utility especially regarding rule-in and rule-out strategies and performances still need to be further investigated and validated to make it fully applicable in clinical settings. We were also restricted for not measuring ceramides levels in a serial pattern and lacking information about the relationships between ceramides levels and time from the onset of ACS. The study was possibly also limited by a potential selection bias for patients with higher risk of ACS during recruitment process from admitted patients rather than patients in emergency departments. In addition, the results found in our study need

to be validated in independent external cohorts. An improvement in the test technique of ceramides, including standardization of test protocol and automated pretreatment of samples is also needed to fulfil the requirement of clinical practice.

Our findings of two ceramides molecules (Cer(d18:1/14:0), Cer(d18:1/22:0)) and one ceramide ratios (Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0)) as diagnostic biomarkers for ACS among patients with suspected symptoms have never been reported before. In addition, only relatively modest correlations between ceramides and lipid profiles including TG, TC, LDL-C, ApoB and ApoE were detected and there was no significant correlation between ceramides with hs-cTnT, NT-proBNP and CRP levels, suggesting that the diagnostic value of ceramides for ACS might be independent of the above laboratory variables. Moreover, AUROC of multi-variable models showed significant improvement of ceramides on traditional risk factors and high-sensitive troponin T. This result provides evidence that distinct ceramide species serve as independent predictors for the risk of ACS, in addition to conventional blood biomarkers such as HDL-C and CRP levels. Ceramides measurement in high-throughput quality-controlled environments is straightforward.[19] By setting up clinical laboratories equipped with robotized sample handling systems and mass spectrometry equipment, it would be feasible to identify patients with chest pain at high cardiovascular risk using our ceramides-based diagnostic model. [19]

Previous clinical research found that elevated ceramide plasma concentrations are associated with coronary plaque vulnerability evaluated by intravascular ultrasound virtual histology and near-infrared spectroscopy.[11] Untargeted lipidomic analyses have identified CAD risk-related ceramide molecules (Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/24:1)) and their ratios with Cer(d18:1/24:0) as potential risk stratifiers for CAD patients.[22] A previous study applying ceramides to the prediction of cardiovascular death from patients with CAD showed that ceramides, independent of other lipid markers and CRP, were significantly associated with CV death.[19] In a recent study including 495 participants with coronary angiography and followed up for 18 years, ceramides were not associated with CAD but were independently associated with major adverse cardiovascular events.[23] A weighted

sum of ceramides including Cer22:0 and Cer24:1 was also reported to be associated with a 2.18-fold higher risk of cardiovascular disease, which was similar with our results.[27] Recent studies showed targeted profiling of ceramides predicted short-term and long-term major adverse cardiac events (MACE).[28 29] Moreover, a consistent increase in ceramide levels and overexpression of 3 enzymes in ceramide biosynthesis were found on rat ischemic myocardium, which is consistent with the elevated plasma levels of ceramides found in our cohort.[29] Nevertheless, no study has identified the diagnostic value of ceramides in patients with chest pain, while we develop models with lipidomic markers for ACS with additional value upon conventional factors and troponin.

Currently, the standard process for the diagnostic assessment of ACS in patients with suspected symptoms relies on clinical evaluation and high-sensitive troponin tests.[4 20] However, high-sensitive troponin assays identify a larger number of patients with elevated troponin results but without a final diagnosis of ACS, making interpretation of test results challenging.[30] The adoption of highly sensitive assays for ACS detection should be accompanied by serial tests and implementation of algorithms, leading to a longer period of observation[31]. In the current settings of Chinese hospitals, self-paying patients with chest pain tend to be over-prescribed by cardiologists with invasive and expensive tests such as coronary angiography. Noteworthily, ceramides levels of patients with UA are higher than those without ACS, which indicates that ceramides may help in identifying patients with UA and higher risk of ACS from populations without significant elevation of cardiac troponins. Moreover, ceramides levels were higher in NSTEMI patients compared to non-ACS ones, indicating a possible role of ceramides in distinguishing between patients with elevated troponins caused by ACS or non-ACS conditions. Nevertheless, the potential roles of ceramides in clinical diagnosis of UA and NSTEMI need further independent investigation and validation.

In conclusion, we have identified ceramides as independent biomarkers associated with ACS and developed a ceramides-based model to evaluate ACS risk among patients with chest pain. The model showed good model discrimination and net reclassification improvement as compared to other models.

Our study characterises ceramides species in Chinese patients with chest pain and demonstrates that ceramides are of diagnostic value and have the potential for detecting ACS, in addition to currently used lipid markers and high-sensitive troponin.

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Conflict of interest: Q-life lab holds patents for the diagnostic use of ceramides for cardiovascular risk determination in China. WG, GX and CX work for Q-life and GX and CX are also shareholders. Other authors declare no conflict of interest.

Patient consent: Obtained.

Ethics approval: This study was approved by the Ethics Committee of Zhongshan Hospital, the Ethics Committee of Tongji Hospital, the Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital, and the Ethics Committee of Minhang Hospital.

Data sharing statement: Data is available with the corresponding author and will be available on request.

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Table 1. Demographics, clinical and lab findings of included patients in total cohort.

Variables	Total cohort (n=2773)	non-ACS (n=2419)	ACS (n=354)	*P*
Age (years)	63.5 (9.6)	63.4 (9.4)	63.8 (10.6)	0.515
Male, n (%)	1894 (68.3)	1634 (67.6)	260 (73.4)	0.031
Diabetes, n (%)	689 (24.8)	600 (24.8)	89 (25.1)	0.943
Hypertension, n (%)	1744 (62.9)	1500 (62.0)	244 (68.9)	0.014
Current Smoker, n (%)	634 (22.9)	523 (21.6)	111 (31.4)	<0.001
TG (mmol/L)	1.46 (1.03-2.10)	1.43 (1.01-2.07)	1.61 (1.16-2.23)	<0.001
TC (mmol/L)	3.78 (3.17-4.59)	3.74 (3.13-4.49)	4.28 (3.51-5.01)	<0.001
HDL-C (mmol/L)	1.09 (0.90-1.30)	1.09 (0.91-1.31)	1.01 (0.88-1.23)	<0.001
LDL-C (mmol/L)	1.93 (1.40-2.61)	1.88 (1.36-2.53)	2.42 (1.77-3.08)	<0.001
ApoA1 (g/L)	1.32 (1.16-1.51)	1.33 (1.17-1.52)	1.24 (1.08-1.43)	<0.001
ApoB (g/L)	0.73 (0.58-0.91)	0.72 (0.57-0.89)	0.84 (0.67-1.03)	<0.001
ApoE (mg/L)	38.0 (30.0-47.0)	37.0 (30.0-46.4)	41.0 (32.0-51.0)	<0.001
Lp(a) (mg/L)	125.0 (53.0-290.3)	123.0 (51.0-282.0)	152.0 (65.0-329.0)	0.023
NTproBNP (ngpg/Lml)	100.0 (44.7-268.1)	87.4 (42.3-220.3)	364.1 (113.4-1264.0)	<0.001
CRP (mg/L)	1.7 (0.7-5.5)	1.5 (0.6-4.8)	5.4 (1.8-13.9)	<0.001
hs-cTnT (ng/L)	9 (5-16)	8 (5-13)	71 (11-474)	<0.001

Data are median (interquartile range) or count (percentage).

TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; ApoE, apolipoprotein E; LP(a), lipoprotein(a); NTproBNP, N-terminal pro-brain natriuretic peptide; CRP, C-reactive protein; hs-cTnT, high-sensitivity cardiac troponin T.

P value for the difference between patients diagnosed as ACS and non-ACS.

Table 2. Plasma levels of ceramides molecules and ratios in total cohort and by ACS status.

Ceramides (μmol/L)	Total cohort (n=2773)	non-ACS (n=2419)	ACS (n=354)	*P*
Cer(d18:1/16:0)	0.203 (0.165-0.248)	0.198 (0.162-0.241)	0.237 (0.203-0.286)	<0.001
Cer(d18:1/18:0)	0.051 (0.037-0.069)	0.049 (0.036-0.065)	0.068 (0.051-0.084)	<0.001
Cer(d18:1/24:0)	2.065 (1.607-2.680)	2.053 (1.592-2.672)	2.164 (1.675-2.757)	0.028
Cer(d18:1/24:1(15Z))	0.583 (0.448-0.767)	0.569 (0.436-0.739)	0.730 (0.557-0.950)	<0.001
Cer(d18:1/14:0)	0.0027 (0.0020-0.0036)	0.0027(0.0020-0.0035)	0.0030 (0.0024-0.0040)	<0.001
Cer(d18:1/20:0)	0.053 (0.040-0.067)	0.051 (0.039-0.065)	0.063 (0.051-0.078)	<0.001
Cer(d18:1/22:0)	0.381 (0.300-0.490)	0.375 (0.294-0.480)	0.416 (0.336-0.540)	<0.001
Cer(d18:0/16:0)	0.010 (0.007-0.014)	0.010 (0.007-0.013)	0.011 (0.008-0.015)	<0.001
Cer(d18:0/18:0)	0.005 (0.003-0.008)	0.005 (0.003-0.007)	0.007 (0.004-0.011)	<0.001
Cer(d18:0/24:0)	0.061 (0.042-0.086)	0.060 (0.042-0.086)	0.068 (0.048-0.094)	<0.001
Cer(d18:0/24:1(15Z))	0.029 (0.020-0.044)	0.028 (0.019-0.041)	0.040 (0.026-0.058)	<0.001
Cer(d18:1/24:1)	0.262 (0.195-0.375)	0.258 (0.192-0.372)	0.298 (0.221-0.386)	<0.001
Ceramides Ratio				
Cer(d18:1/16:0)/Cer(d18:1/24:0)	0.096 (0.080-0.116)	0.095 (0.079-0.114)	0.107 (0.090-0.132)	<0.001
Cer(d18:1/18:0)/Cer(d18:1/24:0)	0.024 (0.019-0.032)	0.023 (0.018-0.031)	0.031 (0.023-0.039)	<0.001
Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0)	0.269 (0.214-0.360)	0.261 (0.211-0.345)	0.321 (0.253-0.426)	<0.001

Data are median (interquartile range).

P value for the difference between patients diagnosed as ACS and non-ACS.

Table 3. Univariate logistic regression showing significant predictors of ACS in patients with chest pain.

Variables	Total Cohort (n=2773) OR (95%CI)	*P*
Age	1.00 (0.99-1.02)	0.476
Gender	1.33 (1.04-1.71)	0.027
Diabetes	1.02 (0.78-1.31)	0.890
Hypertension	1.36 (1.07-1.73)	0.012
Current Smoker	1.66 (1.29-2.11)	<0.001
TG*	1.40 (1.15-1.70)	<0.001
TC*	5.17 (3.30-8.15)	<0.001
HDL-C*	0.44 (0.30-0.66)	<0.001
LDL-C*	3.14 (2.37-4.17)	<0.001
ApoA1*	0.17 (0.09-0.29)	<0.001
ApoB*	2.83 (2.05-4.01)	<0.001
ApoE*	0.93 (0.76-1.17)	0.534
Lp(a)*	1.10 (1.00-1.21)	0.055
NTproBNP*	1.73 (1.61-1.88)	<0.001
CRP*	1.74 (1.59-1.90)	<0.001
hs-cTnT*	2.45 (2.24-2.70)	<0.001
Cer(d18:1/16:0)*	7.53 (5.11-11.17)	<0.001
Cer(d18:1/18:0)*	4.67 (3.55-6.17)	<0.001
Cer(d18:1/24:0)*	1.41 (1.05-1.90)	0.025
Cer(d18:1/24:1(15Z))*	5.09 (3.79-6.87)	<0.001
Cer(d18:1/14:0)*	2.04 (1.59-2.62)	<0.001
Cer(d18:1/20:0)*	4.55 (3.35-6.22)	<0.001
Cer(d18:1/22:0)*	2.36 (1.75-3.18)	<0.001
Cer(d18:0/16:0)*	2.02 (1.61-2.55)	<0.001
Cer(d18:0/18:0)*	2.26 (1.92-2.66)	<0.001
Cer(d18:0/24:0)*	1.49 (1.21-1.84)	<0.001
Cer(d18:0/24:1(15Z))*	2.73 (2.24-3.33)	<0.001
Cer(d18:1/24:1)*	1.57 (1.26-1.96)	<0.001
Cer(d18:1/16:0)/Cer(d18:1/24:0)*	4.11 (2.87-5.89)	<0.001
Cer(d18:1/18:0)/Cer(d18:1/24:0)*	3.84 (2.93-5.05)	<0.001
Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0)*	2.63 (2.08-3.34)	<0.001

Data are odds ratio (OR) (95%CI). *Log transformed before analysis.

TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; ApoE, apolipoprotein E; LP(a), lipoprotein(a); NTproBNP, N-terminal pro-brain natriuretic peptide; CRP, C-reactive protein; hs-cTnT, high-sensitivity cardiac troponin T; Cer, ceramides.

Table 4. Multivariable logistic regression showing significant predictors of ACS in patients with chest pain after adjustment for conventional risk factors.

Baseline variables	Model 1	Model 2	Model 3	Model 4	Model 5
Hypertension	1.37 (1.03-1.83)*	—	1.35 (0.98-1.87)	1.41 (1.06-1.90)*	1.40 (1.01-1.96)*
Current Smoker	1.93 (1.43-2.58)‡	—	1.52 (1.09-2.10)*	1.99 (1.47-2.69)‡	1.58 (1.12-2.21)†
LDL-C§	2.63 (1.91-3.64)‡	—	2.01 (1.42-2.87)‡	2.13 (1.51-3.04)‡	1.64 (1.12-2.41)*
CRP§	1.40 (1.27-1.55)‡	—	1.20 (1.07-1.34)†	1.38 (1.25-1.53)‡	1.16 (1.03-1.31)*
NTproBNP§	1.63 (1.49-1.78)‡	—	1.16 (1.04-1.29)†	1.57 (1.44-1.72)‡	1.07 (0.95-1.19)
hs-cTnT§	—	2.45 (2.24-2.70)‡	2.02 (1.80-2.27)‡	—	2.19 (1.94-2.49)‡
Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0)§	—	—	—	2.96 (2.16-4.07)‡	4.46 (3.11-6.40)‡
Cer(d18:1/14:0)§	—	—	—	1.44 (1.01-2.06)*	1.74 (1.17-2.61)†
Cer(d18:1/22:0)§	—	—	—	2.08 (1.32-3.29)†	2.30 (1.37-3.87)†

Data are odds ratio (OR) (95%CI). §Log transformed before analysis.

Model 1. Included traditional risk factors (Hypertension, current smoker, LDL-C, CRP and NTproBNP levels).

Model 2. Only Included hs-cTnT.

Model 3. Included traditional risk factors, and hs-cTnT.

Model 4. Included traditional risk factors, and ceramides.

Model 5. Included traditional risk factors, hs-cTnT and ceramides.

LDL-C, low-density lipoprotein cholesterol; CRP, C-reactive protein; NTproBNP, N-terminal pro-brain natriuretic peptide; hs-cTnT, high-sensitivity cardiac troponin T; Cer, ceramides.

* $P<0.05$.

† $P<0.01$.

‡ $P<0.001$.

Table 5. Discrimination and reclassification of ACS with various multivariable logistic regression models.

Old model	New model	AUC (95% CI)	*P* Value-AUC	NRI (95% CI)	*P* Value-NRI
Traditional factors*	–	0.78 (0.75-0.81)	–	–	–
hs-cTnT	–	0.80 (0.77-0.83)	–	–	–
Traditional factors*	+hs-cTnT	0.81 (0.78-0.84)	0.220	**0.52 (0.40-0.64)**	**<0.001**
Traditional factors*	+Ceramides†	**0.81 (0.78-0.84)**	**<0.001**	**0.35 (0.23-0.47)**	**<0.001**
Traditional factors* + hs-cTnT	+Ceramides†	**0.87 (0.84-0.89)**	**<0.001**	**0.51 (0.39-0.64)**	**<0.001**

*Traditional risk factors include hypertension, current smoker, LDL-C, CRP and NTproBNP levels.
†Ceramides include Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0), Cer(d18:1/14:0) and Cer(d18:1/22:0).
LDL-C, CRP, NT-proBNP, hs-cTnT and ceramides levels are log transformed before analysis.
hs-cTnT, high-sensitivity cardiac troponin T; LDL-C, low-density lipoprotein cholesterol; CRP, C-reactive protein; NT-proBNP, N-terminal pro-brain natriuretic peptide.
P values-AUC and *P* values-NRI are for the difference between old models and new models. Bold used to highlight those *P* values<0.05.

Figure 1: Flow chart.

Figure 2: Receiver operating characteristic (ROC) curves of multi-variable models for the discrimination of ACS.

(A) Traditional risk factors (hypertension, current smoker, LDL-C, CRP and NT-proBNP) and high-sensitive troponin are included in the model. (B) Traditional risk factors (hypertension, current smoker, LDL-C, CRP and NT-proBNP), high-sensitive troponin, Cer(d18:1/14:0), Cer(d18:1/22:0) and Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0) are included in the model.